The purification and characterization of 4-ethylphenol methylenehydroxylase, a flavocytochrome from *Pseudomonas putida* JD1

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The enzyme 4-ethylphenol methylenehydroxylase was purified from *Pseudomonas putida* JD1 grown on 4-ethylphenol. It is a flavocytochrome *c* for which the M_r was found to be 120 000 by ultracentrifuging and 126 000 by gel filtration. The enzyme consists of two flavoprotein subunits each of M_r 50 000 and two cytochrome *c* subunits each of M_r 10 000. The redox potential of the cytochrome is 240 mV. Hydroxylation proceeds by dehydrogenation and hydration to give 1-(4'-hydroxyphenyl)ethanol, which is also dehydrogenated by the same enzyme to 4-hydroxyacetophenone. The enzyme will hydroxylate *p*-cresol but is more active with alkylphenols with longer-chain alkyl groups. It is located in the periplasm of the bacterium.

INTRODUCTION

The catabolism of 4-ethylphenol by *Pseudomonas putida* JD1 proceeds by hydroxylation of the methylene group of the ethyl side chain, to give 1-(4'-hydroxyphenyl)ethanol, followed by dehydrogenation to the ketone, 4-hydroxyacetophenone (Darby *et al.*, 1987). To this point the pathway parallels one of two previously reported for the metabolism of the more common alkylphenol, *p*-cresol (4-methylphenol), in which hydroxylation yields 4-hydroxybenzylalcohol and further oxidation gives 4-hydroxybenzaldehyde (Dagley & Patel, 1957). Thereafter the pathways diverge.

The initial enzyme for *p*-cresol metabolism, *p*-cresol methylhydroxylase, has been isolated from several bacteria (Hopper, 1988), and the most thoroughly studied example is the A hydroxylase from *Ps. putida* N.C.I.B. 9869 (McIntire *et al.*, 1985). It is an O_2 -independent hydroxylase, deriving the hydroxy group from water and is thought to act by dehydrogenation of the substrate to a quinone methide followed by hydration. It will also catalyse the dehydrogenation of the alcohol produced to the aldehyde. The enzyme is a flavocytochrome of M_r 115 000 consisting of two flavoprotein subunits each of M_r 50 000 and two cytochrome *c* subunits each of M_r 8000 (McIntire *et al.*, 1986). The substrate-specificity of this enzyme is sufficiently broad to allow it to hydroxylate the methylene group of 4-ethylphenol and also to convert the product into 4-hydroxyacetophenone (McIntire *et al.*, 1984a), the first two steps of the 4-ethylphenol pathway in *Ps. putida* JD1. This latter organism will also grow on *p*-cresol, resulting in induction of an O_2 -independent *p*-cresol methylhydroxylase. However, this is not the enzyme used for growth on 4-ethylphenol. Although of a similar type, the electrophoretic properties and specificity in crude extract of the hydroxylase induced by growth on 4-ethylphenol or 4-n-propylphenol show that this is a different enzyme (Reeve *et al.*, 1988). The purification and some properties of this 4-ethylphenol methylenehydroxylase are described here.

MATERIALS AND METHODS

Growth of organism

Ps. putida JD1 was maintained by growth at 30 °C on nutrient agar slopes followed by storage at 4 °C. Cells for enzyme purification work were grown in liquid medium containing (per l) 4.8 g of Na_2HPO_4 , 2.4 g of KH_2PO_4 , 2.0 g of NH_4Cl , 0.5 g of 4-ethylphenol and 4.0 ml of salts solution (Rosenberger & Elsdon, 1960).

Large batches were grown in a 12-litre Microferm MF-114 fermenter (New Brunswick Instrument Co., New Brunswick, NJ, U.S.A.) at 30 °C, with an aeration rate of 4 litres/min and a stirring rate of 250 rev./min. The fermenter was inoculated with 1 litre of culture grown at 30 °C in a 2-litre Erlenmeyer flask on an orbital shaker. Growth was monitored by measuring the absorbance of the culture at 540 nm. Further additions of 4-ethylphenol equivalent to 0.03 % (w/v) were made when necessary. Yields were increased by inoculating the 12-litre culture into 30 litres of medium in a vessel modified to fit the fermenter and continuing growth under non-sterile conditions for a few hours.

Bacteria were harvested by using an Alfa Laval continuous-flow centrifuge and washed by resuspension in 21 mM-sodium/potassium phosphate buffer, pH 7.1, followed by centrifuging at 34 000 g for 20 min. The cell paste was stored at –20 °C.

Enzyme assay

4-Ethylphenol methylenehydroxylase was assayed as described for *p*-cresol methylhydroxylase (McIntire *et al.*, 1985) but with 0.66 mM-4-ethylphenol as substrate instead of *p*-cresol. One unit of enzyme activity is equivalent to the oxidation of 1 μ mol of 4-ethylphenol/min at 25 °C.

For kinetic studies horse heart cytochrome *c* was used as the reoxidizing substrate and the reaction was monitored at 25 °C by measuring the change in absorbance at 550 nm ($\Delta\epsilon_{red.-ox.}$ 21.1 mM⁻¹·cm⁻¹). Initial

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rates were measured for reaction mixtures consisting of 1 ml of 10 mM-Tris/HCl buffer, pH 7.6, containing cytochrome *c* (in the range 10–50 μ M), various concentrations of phenolic substrate and 9.68 pmol of enzyme. The data were analysed to obtain $V_{\max}$ and K_m values by using a non-linear-regression-analysis computer program. Assays to study the extent of oxidation of substrates were done with an oxygen electrode (oxygen monitor model 53; Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). The reaction mixture at 30 °C contained in 3 ml of 42 mM-sodium/potassium phosphate buffer, pH 7.5, 2 μ mol of phenazine methosulphate, 0.6 μ mol of substrate and enzyme.

Enzyme purification

Crude cell extract was prepared by thawing and resuspending frozen cells in 21 mM-sodium/potassium phosphate buffer, pH 7.1 (2 ml/g wet wt.), followed by two passages through a precooled French pressure cell with a pressure difference at the orifice of 138 MPa. Cell debris was removed by centrifuging at 34000 *g* for 20 min at 4 °C. All purification steps were performed at 4 °C.

Crude extract from 80–90 g of cells was loaded on to a DE-52 DEAE-cellulose column (17 cm $\times$ 5 cm diam.) pre-equilibrated with 21 mM-sodium/potassium phosphate buffer, pH 7.1. The column was washed with 2 column volumes of buffer and then eluted with buffer containing 0.25 M-KCl. The enzyme corresponded to an orange-red band on the column, and this was collected as a single fraction of 320 ml, which was dialysed for 18 h against 10 litres of buffer.

The dialysed enzyme was loaded on to a second DE-52 DEAE-cellulose column (35 cm $\times$ 2.5 cm diam.) equilibrated as before. The column was washed with 1 column volume of buffer and then the enzyme was eluted with a linear gradient of 0–0.3 M-KCl in 1800 ml of buffer; 10 ml fractions were collected. Fractions 42–58 were pooled and dialysed for 18 h against 10 litres of 2 mM-potassium phosphate buffer, pH 7.2.

The dialysed enzyme was loaded on to a hydroxyapatite column (10 cm $\times$ 2.5 cm diam.) equilibrated with 2 mM-potassium phosphate buffer, pH 7.2. The column was washed with 1 column volume of buffer, and then the enzyme was eluted with a linear gradient of 2–300 mM-potassium phosphate buffer, pH 7.2, in 500 ml; 5 ml fractions were collected. Fractions 40–56 were pooled, dialysed for 18 h against 10 litres of distilled water and then freeze-dried. The enzyme was stored at –20 °C.

When examined by polyacrylamide-gel electrophoresis at this stage the enzyme contained only one very minor contaminant. For some experiments this was removed by f.p.l.c. The enzyme was injected in 0.5 mg portions dissolved in 21 mM-sodium/potassium phosphate buffer, pH 7.1, on to a Mono Q (HR 5/5) column (Pharmacia, Milton Keynes, Bucks., U.K.). It was eluted with a linear gradient of 0–0.3 M-KCl in 20 ml of buffer at a flow rate of 1 ml/min. The enzyme peak was eluted at 13 min. The hydroxylase fractions from several runs were pooled, dialysed against distilled water and then freeze-dried. The enzyme was stored at –20 °C.

Protein assays

Protein was determined by the modified biuret method of Gornall *et al.* (1949) or by the tannic acid turbidometric method (Mejbaum-Katzenellenbogen & Dobryszczyka, 1959). In both cases bovine serum albumin was used as

the standard. Protein being eluted from columns was monitored by absorbance at 280 nm.

Isolation of flavin peptides

Digestion of 10 mg of purified hydroxylase with 2 mg of pepsin and subsequent isolation of the peptides were performed by using the procedure described by McIntire *et al.* (1980). The freeze-dried peptides were separated by f.p.l.c. on a Mono Q column with a linear gradient of 0–0.3 M-KCl in 20 ml of 21 mM-sodium/potassium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min; 1 ml fractions were collected.

Spectra

Visible- and u.v.-absorption spectra were recorded on a Pye-Unicam SP.8-150 spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer LS-5B luminescence spectrometer.

Polyacrylamide-gel electrophoresis

Electrophoresis on polyacrylamide slab gels (17 cm $\times$ 17 cm $\times$ 0.2 cm) was used to assess protein purity and, in the presence of SDS, to study subunit structure by the procedure of Laemmli (1970). After electrophoresis gels were stained with 0.25 % (w/v) Coomassie Brilliant Blue R-250 in methanol/water/acetic acid (5:4:1, by vol.) for 2 h and then destained in ethanol/water/acetic acid (3:6:1, by vol.).

Isoelectric focusing

Isoelectric focusing was performed on thin-layer 5 % polyacrylamide gels with Ampholines in the pH range 3.5–10.0. After focusing a 1 cm strip was removed from the edge of the gel and cut into 0.5 cm segments, which were each soaked overnight in 1.0 ml of water. The pH gradient was then determined by measuring the pH of each sample.

Determination of M_r

Sedimentation-equilibrium experiments for M_r determinations were performed in a Beckman Spinco model E analytical ultracentrifuge equipped with Rayleigh interference optics. Before being centrifuged samples were dialysed against 50 mM-Tris/HCl buffer, pH 7.5, for 24 h at 4 °C. Meniscus-depletion experiments were carried out according to the method of Yphantis (1964) at 19160 rev./min for 24 h at 20 °C with an initial protein concentration of 0.45 mg/ml. The attainment of equilibrium was determined experimentally by measuring fringe displacements of given radial positions. A value of 0.725 cm³/g was assumed for the partial specific volume, $\bar{v}$, of the protein.

The M_r of the native protein was also determined by gel filtration on a Sephacryl S-200 column (87 cm $\times$ 2.5 cm diam.) equilibrated with 0.1 M-Tris/HCl buffer, pH 7.5. The column was calibrated with fumarase (pig heart; M_r 200000), alcohol dehydrogenase (yeast; M_r 150000), lactate dehydrogenase (rabbit muscle; M_r 140000), alkaline phosphatase (calf intestine; M_r 100000), malate dehydrogenase (pig heart; M_r 62000) and ovalbumin (M_r 45000).

Subunit M_r values were determined by electrophoresis on SDS/polyacrylamide gels calibrated with bovine serum albumin (M_r 66000), ovalbumin (M_r 45000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 29000), trypsinogen (M_r 24000),

trypsin inhibitor (M_r 20 100), α -lactalbumin (M_r 14 200) and the cytochrome *c* subunit of *p*-cresol methylenedehydroxylase (M_r 8600).

Isolation and chromatography of reaction products

Reaction mixtures were acidified to pH 1 with 2 M-HCl and extracted three times with 20 ml of diethyl ether. The pooled ether extracts were dried over anhydrous Na_2SO_4 , concentrated to 5 ml on a rotary evaporator and then evaporated to dryness under a stream of N_2 . The products were examined by t.l.c. on precoated silica-gel GHLF plates (Analtech, Newark, NJ, U.S.A.) with solvent A [toluene/dioxan/acetic acid (90:25:4, by vol.)] (Pastuska, 1961)] and solvent B [light petroleum (b.p. 40–60 °C)/ethyl formate/propionic acid (70:30:15.4, by vol.)]. Phenolic compounds were detected by fluorescence quenching when viewed under short-wavelength u.v. light and by spraying with Folin–Ciocalteu phenol reagent followed by exposure to NH_3 .

Measurement of redox potential

The midpoint potential of the cytochrome in the enzyme was determined by the procedure of Dutton (1978) with a Perkin–Elmer model 356 dual-wavelength spectrophotometer. Enzyme was titrated with $\text{Na}_2\text{S}_2\text{O}_4$ and $\text{K}_3\text{Fe}(\text{CN})_6$ in a stirred anaerobic cuvette. The enzyme, sufficient to give an absorbance change at 550 nm of 0.2 between fully oxidized and fully reduced forms, was dissolved in 50 mM-Mops/KCl buffer, pH 7.0, and placed in the cuvette together with 5 μl each of the mediators, diaminodurene (11 mg/ml; $E_{m,7}$ 0.26 V), phenazine ethosulphate 3 mg/ml; $E_{m,7}$ 0.055 V) and phenazine methosulphate (3 mg/ml; $E_{m,7}$ 0.08 V). Anaerobic conditions were maintained by a flow of Ar gas purged of O_2 by passage through Fieser's solution. The change in absorbance at 550 nm relative to an isosbestic point at 540 nm was monitored, as was the potential in the cuvette relative to a standard calomel electrode.

Fractionation of cells

The procedure for cell fractionation was essentially that of Wood (1978). Freshly harvested cells (approx. 1 g) were resuspended in 40 ml of 40 mM-Tris/HCl buffer, pH 8.0, containing 4 mM-EDTA and 0.5 M-sucrose at 30 °C. The cell suspension was incubated at 30 °C with 10 mg of lysozyme for 1.5 min, after which 0.8 ml of 0.5 M- MgCl_2 was added and incubation continued for 30 min. The suspension was centrifuged at 15000 *g* for 20 min at 30 °C, giving the supernatant periplasmic fraction

and a pellet of spheroplasts. The spheroplasts were burst by resuspension in 20 ml of 10 mM-Tris/HCl buffer, pH 8.0, containing 2 mM-EDTA and incubation at 30 °C for 30 min, after which 0.4 ml of 0.5 M- MgCl_2 and a trace of DNAase were added. Incubation was continued for a further 15 min, and the suspension was then centrifuged at 15000 *g* for 30 min at 30 °C to give a supernatant cytoplasmic fraction and a pellet of membrane fragments. The pellet was resuspended in 5 ml of 40 mM-Tris/HCl buffer, pH 8.0.

Isocitrate dehydrogenase, a cytoplasmic marker, was assayed as described by Hopper *et al.* (1985). Succinate dehydrogenase, a membrane marker, was assayed spectrophotometrically by monitoring the decrease in A_{600} of a reaction mixture at 25 °C containing, in 3 ml of 0.1 M-sodium/potassium phosphate buffer, pH 7.0, 10 μmol of KCN, 4 μmol of sodium succinate, 0.17 μmol of 2,6-dichlorophenol-indophenol and enzyme.

Chemicals

4-Ethylphenol was obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). 4-n-Propylphenol, 4-n-butylphenol, 4-n-pentylphenol, 4-n-heptylphenol, 4-n-nonylphenol, 5-indanol, eugenol and 4-hydroxyacetophenone were obtained from Lancaster Synthesis (Morecambe, Lancs., U.K.). Fumarase, alcohol dehydrogenase, malate dehydrogenase, lysozyme, pepsin, SDS/polyacrylamide-gel M_r calibration kits and phenazine ethosulphate were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Phenazine methosulphate, alkaline phosphatase, 2,6-dichlorophenol-indophenol, *p*-cresol and diaminodurene were from BDH Chemicals (Poole, Dorset, U.K.). Coomassie Brilliant Blue R-250 was from Serva Feinbiochemica (Heidelberg, W. Germany). Bio-Gel HTP hydroxyapatite was from Bio-Rad Laboratories (Watford, Herts., U.K.). 1-(4'-Hydroxyphenyl)ethanol was prepared by the reduction of 4-hydroxyacetophenone with NaBH_4 .

RESULTS

Purification of enzyme

The details of a typical purification of 4-ethylphenol methylenedehydroxylase from *Ps. putida* JD1 grown on 4-ethylphenol are summarized in Table 1. Electrophoresis on polyacrylamide gels at this stage showed one major band when stained with Coomassie Brilliant Blue corresponding to the single band seen when a gel was stained for enzyme activity. A minor inactive band seen

Table 1. Summary of the purification of 4-ethylphenol methylenedehydroxylase from *Ps. putida* JD1

The hydroxylase was purified from 87 g of cells grown on 4-ethylphenol as described in the Materials and methods section. One unit of enzyme is that amount which will oxidize 1 μmol of substrate/min.

Purification step	Volume (ml)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	300	14625	0.17	100	1
1st DEAE-cellulose chromatography	318	6599	0.27	73	1.6
2nd DEAE-cellulose chromatography	170	701	1.78	50	10.5
Hydroxyapatite chromatography	83	133	8.98	48	53.0

Fig. 1. Visible-absorption spectra of oxidized (-----) and reduced (—) 4-ethylphenol methylenedehydroxylase

A solution of 0.45 mg of enzyme/ml of 50 mM-Tris/HCl buffer, pH 7.5, was either oxidized with $K_3Fe(CN)_6$ or reduced with $Na_2S_2O_4$.

only on heavily loaded gels represented only a very small proportion of the total protein and could be removed by f.p.l.c.

The purified hydroxylase was an orange-red colour. It required no stabilizing agents and retained greater than 90% of its activity after being freeze-dried and stored at $-20^\circ C$ for several months.

Absorption spectra

The u.v.-visible-absorption spectra of the oxidized and the reduced enzyme are shown in Fig. 1. The absorption maxima of 550 nm, 520 nm and 414 nm in the reduced spectrum are typical of a *c*-type cytochrome, as also are the shift and lowering in intensity of the 414 nm peak and the replacement of the other two peaks by a single broad peak when the enzyme is oxidized. In the oxidized spectrum the shoulder that appears at about 450 nm is indicative of the presence of flavin. These spectral characteristics are similar to those of the flavocytochrome *c* *p*-cresol methylhydroxylase (Hopper & Taylor, 1977).

Although the enzyme was reduced with dithionite for Fig. 1, reduction of oxidized enzyme to give the same spectrum can also be achieved by addition of substrate, 4-ethylphenol.

M_r and subunit structure of enzyme

Gel filtration of the purified enzyme on a calibrated Sephacryl S-200 column gave an M_r of 126 000. A value of 120 000 was given by meniscus-depletion sedimentation-equilibrium studies in the ultracentrifuge.

Polyacrylamide-gel electrophoresis in the presence of SDS of enzyme purified by a final f.p.l.c. step gave rise to two protein bands equivalent to M_r 50 000 and M_r 10 000. When a gel was stained for haem by the method of Goodhew *et al.* (1986) the band at M_r 10 000 was positive, indicating that this is the cytochrome component of the enzyme. This was confirmed by increasing the protein load on such a gel, when the band at M_r 10 000 could be seen to have a red colour and the band at M_r 50 000 was a yellow colour.

A ratio of haem to molecule of enzyme of 1.98:1 was calculated from the absorbance of the reduced enzyme at 550 nm by using ϵ 31.18 $mm^{-1} \cdot cm^{-1}$ for the cytochrome *c* of *p*-cresol methylhydroxylase (McIntire *et al.*, 1985).

Identification of flavin

The similarities in spectral and other properties between this enzyme and the flavocytochrome *p*-cresol methylhydroxylase, together with the evidence from electrophoresis, suggest that flavin is present as a prosthetic group. However, unlike the *p*-cresol methylhydroxylase A from *Ps. putida* N.C.I.B. 9869, it was not possible to separate the flavin-containing and cytochrome subunits by isoelectric focusing for independent study. Isoelectric focusing gave a single protein band at pI 4.7. Neither was it possible to remove the cytochrome subunit with acid acetone, as had been successful for *p*-cresol methylhydroxylase (Keat & Hopper, 1978a). Attempts to demonstrate the presence of flavin by releasing it from the enzyme by heat or trichloroacetic acid precipitation were also unsuccessful. The presence of flavin was, however, confirmed by isolation of a flavin-containing peptide after peptic digestion of the enzyme. The purified hydroxylase was treated with pepsin as described by McIntire *et al.* (1980). The resulting peptides were separated by f.p.l.c. on a Mono Q column. The absorption spectrum of fraction 14 (Fig. 2) was typical of that for a flavoprotein, whereas fraction 8 gave a spectrum for a cytochrome. With the use of riboflavin for comparison (Edmondson *et al.*, 1978) the fluorescence of the flavin-containing peptide was less than 30% of that for an equimolar solution of riboflavin, and the fluorescence excitation spectrum (Fig. 2) showed a hypsochromic shift of the 375 nm peak of riboflavin to 350 nm. Such observations are indicative of covalent binding of the flavin to protein.

Treatment of the flavin-containing peptide with nucleotide pyrophosphatase, which catalyses the hydrolysis of FAD to FMN, resulted in a 4-fold increase in fluorescence.

Redox potential of the cytochrome

The redox potential of the cytochrome *c* in the holoenzyme was determined from a potentiometric titration. The results were consistent with a single electron transfer and gave a midpoint redox potential of 240 mV.

Identification of the products from 4-ethylphenol

4-Ethylphenol was oxidized by the purified enzyme by incubation of a reaction mixture containing, in 10 ml of 50 mM-Tris/HCl buffer, pH 7.6, 30 μ mol of 4-ethylphenol, 20 μ mol of phenazine methosulphate and 1.2 mg of 4-ethylphenol methylenedehydroxylase, for 2 h at $30^\circ C$. The products were extracted and examined by t.l.c. with solvents A and B as described in the Materials and

Fig. 2. U.v.-visible-absorption spectrum and (inset) the fluorescence excitation spectrum of the flavin-containing peptide from 4-ethylphenol methylenedehydroxylase

Both spectra are of a solution of the flavin-containing peptide dissolved in 21 mM-sodium/potassium phosphate buffer, pH 7.1. The peptide was obtained by digestion of the hydroxylase with pepsin and isolated by f.p.l.c. For the fluorescence excitation spectrum (inset) the emission wavelength was 525 nm.

methods section. Two products were observed: the major one corresponded in both solvents to authentic 4-hydroxyacetophenone and the minor one to authentic 1-(4'-hydroxyphenyl)ethanol (Table 2). No starting substrate was detected.

In a similar experiment with 1-(4'-hydroxyphenyl)-ethanol as the substrate, the product isolated was 4-hydroxyacetophenone together with some unchanged substrate.

Substrate-specificity

The substrate-specificity of the hydroxylase was investigated with a number of aromatic compounds in the spectrophotometric assay. Those that gave activity were also used in the oxygen-monitor assay to measure the stoichiometry of the reaction, thus ensuring that a substantial proportion of the substrate was oxidized and that apparent activity was not due to small amounts of contaminants. A list of substrates and their relative activities with the hydroxylase in the standard assay are given in Table 3. Compounds not active with the enzyme included 2,3-xyleneol, 2,5-xyleneol, 2,6-xyleneol, 3,4-xyleneol, 3,5-xyleneol, *o*-cresol, 4-methoxyphenol, 4-isopropylphenol and tyrosine.

Steady-state kinetics

The main objective of the steady-state kinetic experiments was to allow a comparison of *p*-cresol and 4-

Table 2. Identification by t.l.c. of products from the action of 4-ethylphenol methylenedehydroxylase on 4-ethylphenol and 1-(4'-hydroxyphenyl)ethanol

Compound	R_F	
	Solvent A	Solvent B
4-Ethylphenol	0.59	0.95
1-(4'-Hydroxyphenyl)ethanol	0.34	0.44
4-Hydroxyacetophenone	0.44	0.54
4-Vinylphenol	0.56	0.50
Products from:		
4-Ethylphenol	0.34, 0.44	0.45, 0.55
1-(4'-Hydroxyphenyl)ethanol	0.44	0.55

Table 3. Relative activities of 4-ethylphenol methylenedehydroxylase towards various phenolic substrates

Each substrate was investigated in the phenazine methosulphate/2,6-dichlorophenol-indophenol spectrophotometric assay with 25 μ g of the hydroxylase and 2 μ mol of substrate

Substrate	Activity relative to that with 4-ethylphenol (100 %)
<i>p</i> -Cresol (4-methylphenol)	44
4-n-Propylphenol	112
4-n-Butylphenol	114
4-n-Pentylphenol	106
4-n-Heptylphenol	52
4-n-Nonylphenol	14
1-(4'-Hydroxyphenyl)ethanol	30
2,4-Xyleneol	29
<i>p</i> -Hydroxybenzyl alcohol	14
4-Allyl-2-methoxyphenol (eugenol)	6
5-Indanol	58

ethylphenol as substrates for the hydroxylase by the k_{cat}/K_m ratios for these two compounds. Preliminary kinetic studies with the phenazine methosulphate/2,6-dichlorophenol-indophenol-linked assay, with a high 4-ethylphenol concentration and various phenazine methosulphate concentrations, showed that, like *p*-cresol methylhydroxylase, this enzyme too was inhibited by phenazine methosulphate concentrations above 5 mM. The K_m for phenazine methosulphate was greater than 10 mM, higher than the 4.48 ± 0.41 mM given by *p*-cresol methylhydroxylase (McIntire *et al.*, 1987). A full two-substrate kinetic analysis with this assay proved difficult, with variable results, and the experiments were performed instead with horse heart cytochrome *c* as the reoxidizing substrate. The double-reciprocal primary plots of the results gave almost parallel lines with *p*-cresol or 4-ethylphenol, and the K_m and k_{cat} values obtained from the two-substrate analysis are given in Table 4.

Cellular location of hydroxylase

Fresh cells of *Ps. putida* JD1 grown on 4-ethylphenol were treated with lysozyme and EDTA to give periplasmic, cytoplasmic and membrane fractions. The success of the fractionation procedure was assessed by

Table 4. Steady-state kinetic parameters for 4-ethylphenol methylenedehydroxylase

Enzyme assays were carried out at 25 °C in 10 mM-Tris/HCl buffer, pH 7.6 (I 0.01), with cytochrome *c* concentrations in the range 2–50 μ M and various 4-ethylphenol (0.019–0.838 mM) and *p*-cresol (3–8 mM) concentrations. The concentration of hydroxylase was 9.68 nM.

Substrate (S)	K_m (μ M)		k_{cat} (s^{-1})	$10^{-4} \times k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)	
	S	Cytochrome <i>c</i>		S	Cytochrome <i>c</i>
4-Ethylphenol	55.4 $\pm$ 2.3	43.2 $\pm$ 4.1	194 $\pm$ 3	350	449
<i>p</i> -Cresol	3010 $\pm$ 33	7.02 $\pm$ 0.3	29.4 $\pm$ 1.1	0.98	419

Table 5. Distribution of 4-ethylphenol methylenedehydroxylase and two marker enzymes in *Ps. putida* JD1

Periplasmic, cytoplasmic and membrane fractions were prepared by treatment of whole cells with lysozyme and EDTA. The activities of the two marker enzymes and the hydroxylase were assayed as described in the Materials and methods section.

Fraction	Recovery of enzyme (% of total)		
	Isocitrate dehydrogenase	Succinate dehydrogenase	4-Ethylphenol methylenedehydroxylase
Periplasm	22	0	83
Cytoplasm	78	0	13
Membrane	0	100	4

assaying isocitrate dehydrogenase as a cytoplasmic marker and succinate dehydrogenase as a membrane marker. The 4-ethylphenol methylenedehydroxylase was found predominantly in the periplasm (Table 5).

DISCUSSION

The 4-ethylphenol methylenedehydroxylase was purified 53-fold and represents about 2% of the total soluble protein of the cell. Its spectral properties (Fig. 1) showed clearly the presence of cytochrome, which, from the position of its α -band at 550 nm was taken to be a *c*-type. The presence of flavin, suggested by the shoulder at 450 nm in the oxidized spectrum and by the yellow colour of one of the bands seen after SDS/polyacrylamide-gel electrophoresis, was confirmed by the isolation of a flavin-containing peptide by f.p.l.c. after treatment of the enzyme with pepsin (Fig. 2). The lack of success in releasing the flavin from the enzyme, the fluorescence quenching and the hypsochromic shift of the 375 nm peak to 350 nm in the fluorescence excitation spectrum all indicate covalent binding of the flavin to the protein, and the increase in fluorescence on treatment of the flavin-containing peptide with pyrophosphatase shows the flavin to be FAD. A quantitative measurement of the amount of flavin in the enzyme has not been made, but measurements of M_r of the native enzyme and of the subunits suggest the presence of two flavin-containing subunits and two cytochrome subunits in each molecule of the hydroxylase. The M_r of the enzyme by gel filtration was 126 000 and from ultracentrifuge studies was 120 000.

SDS/polyacrylamide-gel electrophoresis gave a red band, the cytochrome subunit, at M_r 10 000 and a yellow band, the flavin-containing subunit, at M_r 50 000. This assignment of subunit structure also agrees with the stoichiometry of 2:1 for the cytochrome content per molecule of enzyme ascertained from the absorption spectrum.

In most of these properties the 4-ethylphenol methylenedehydroxylase resembles *p*-cresol methylhydroxylase as described in the Introduction. The enzyme was released from the cell by treatment with lysozyme and EDTA in iso-osmotic solution, and by comparison with marker enzyme appears to be located in the periplasm (Table 5). In this property and in the midpoint potential of 240 mV for its cytochrome component it again resembles *p*-cresol methylhydroxylase (Hopper *et al.*, 1985; Hopper, 1983). The flavin-containing and cytochrome subunits of 4-ethylphenol methylenedehydroxylase could not be separated for individual examination by isoelectric focusing as has been done for *p*-cresol methylhydroxylase A from *Ps. putida* N.C.I.B. 9869 (McIntire & Singer, 1982). However, this is not a characteristic of all *p*-cresol methylhydroxylases, and *p*-cresol methylhydroxylase B (also from *Ps. putida* N.C.I.B. 9869) cannot be resolved in this way, presumably because of the closeness of the isoelectric points of the two types of subunit (Koerber *et al.*, 1985). The pI for 4-ethylphenol methylenedehydroxylase of 4.7 is slightly lower than those for *p*-cresol methylhydroxylases, which lie between 5.0 and 5.8 (McIntire *et al.*, 1984b).

The major difference between 4-ethylphenol methylenedehydroxylase and *p*-cresol methylhydroxylase lies in the former's specificity in that it is better adapted to the hydroxylation of 4-alkylphenols with longer-chain alkyl groups. This is apparent from the steady-state kinetics (Table 4), where a full two-substrate analysis was performed with both *p*-cresol and 4-ethylphenol to allow a comparison of k_{cat}/K_m values. These are 0.98×10^4 and $350 \times 10^4 \cdot M^{-1} \cdot s^{-1}$ respectively for 4-ethylphenol methylenedehydroxylase compared with 870×10^4 and $7.55 \times 10^4 \cdot M^{-1} \cdot s^{-1}$ respectively for *p*-cresol methylhydroxylase (McIntire *et al.*, 1987). The K_m for *p*-cresol was also much higher than that for 4-ethylphenol. The kinetics indicate a similar mechanism for the two enzymes and their k_{cat} values are of the same order.

Longer-chain 4-alkylphenols are also substrates for the 4-ethylphenol methylenedehydroxylase, whereas they give little or no activity with *p*-cresol methylhydroxylase. These have only been tested at a single concentration and relative rates were determined (Table 3), but there was activity up to an alkyl side chain of C_9 , which was the longest tested. Another contrast with *p*-cresol

methylhydroxylase is the inability of 4-ethylphenol methylenedehydroxylase to oxidize 3,4-xyleneol.

As with *p*-cresol methylhydroxylase, it is proposed that the reaction proceeds by dehydrogenation via a quinone methide intermediate. All substrates for the enzyme had a hydrogen-bearing carbon *para* to a hydroxy group, as required for this mechanism. Thus the initial product, 1-(4'-hydroxyphenyl)ethanol, is also a substrate, and 4-hydroxyacetophenone was identified as the product from either this compound or from 4-ethylphenol. Both reactions are of physiological importance in the degradation of 4-ethylphenol by *Ps. putida* JD1, whereas the significance of the second dehydrogenation by *p*-cresol methylhydroxylase, the conversion of *p*-hydroxybenzylalcohol into *p*-hydroxybenzaldehyde, is uncertain because of the presence of an NAD-linked alcohol dehydrogenase in cell extracts (Keat & Hopper, 1978*b*). No 1-(4'-hydroxyphenyl)ethanol dehydrogenase other than 4-ethylphenol methylenedehydroxylase has been found in *Ps. putida* JD1. This type of hydroxylase does provide a mechanism for the anaerobic metabolism of alkyl groups attached to the benzene nucleus and, although the organism used here was grown aerobically, there is a growing number of reports of the anaerobic metabolism of alkylphenols by bacteria where such a mechanism may be operative (Bossert & Young, 1986; Smolenski & Sufita, 1987).

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